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Differentiation of Cultured Epithelial Cells: Response to Toxic Agents

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Cell culture systems are instrumental in elucidating regulation of normal function and mechanisms of its perturbation by toxic substances. To this end, three applications of epithelial cells cultured with 3T3 feeder layer support are described. First, treatment of the premalignant human epidermal keratinocyte line SCC-12F2 with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate suppressed cell growth and differentiation. This agent produced a biphasic growth response greatly inhibiting cell growth at 1 to 10 nM, but much less above 100 nM. Expression of the differentiated functions involucrin and transglutaminase was found to be inhibited markedly at concentrations above 10 nM. Second, 3-methylcholanthrene toxicity was surveyed in a variety of rat epithelial cell types. The two most sensitive to growth inhibition were epidermal and mammary epithelial cells, while those from bladder, prostate, thyroid, and endometrium were insensitive to growth inhibition. Great differences were evident even among those cells derived from stratified squamous epithelia (epidermal, esophageal, vaginal, forestomach) despite their expression of aryl hydrocarbon hydroxylase activities to similar degrees. Finally, expression of estrogen receptors in rat endometrial cells was shown to be stimulated by the cAMP-elevating agent forskolin. Maximal stimulation of 3- to 6-fold occurred in 6 hr, compatible with a requirement for protein synthesis. Although expressing keratinocyte character (transglutaminase activity and envelope forming ability), the cells thus retain some hormonal character that may be modulated by cAMP-dependent kinase activity. Pursuit of such results will aid in understanding differences in response among cell types and species, in elucidating mechanisms of action of known toxic substances and, ultimately, in predicting toxicity of less well understood agents.

Introduction

Despite the variety of toxicity testing methods now available, vexing uncertainties persist in estimating the risk to humans posed by environmental contaminants. Major problems arise in extrapolating test results among species, from high to low doses, and to chemical mixtures. Advances in dealing with these uncertainties are anticipated to result from better mechanistic understanding of the interactions of agents with target cells. In turn, as differentiated functions of target cells become better understood, the actions of toxic agents will be more readily elucidated. An added incentive to examining the perturbation of cellular processes is that such study is likely to provide further insight into normal regulatory and developmental phenomena, some of which are poorly understood at present.

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Inasmuch as they are common targets of environmental agents, keratinocytes are especially appropriate for this type of investigation. Considerable information has accumulated on the differentiated functions of this cell type (*1*), which can now be serially cultivated from humans and various animal species. Among the useful markers of keratinocyte maturation available for study (including filaggrin and the keratin intermediate filament proteins) are involucrin and keratinocyte transglutaminase. The latter is a membrane-bound enzyme responsible for cross-linking involucrin (*2,3*) and certain other proteins (*4,5*) into insoluble cornified envelope structures (*6,7*). Involucrin is found throughout the primate order (*8*) but has been difficult to identify in other animals, although evidence has been reported recently for a related protein in bovine epidermis (*9*). Keratinocyte transglutaminase is distinct from the tissue transglutaminase found in many cell types in its regulation and its biochemical and immunochemical properties (*7,10,11*). The relationships of these enzymes to the soluble transglutaminases purified from epidermis and hair follicle are uncertain (*12–14*).

One approach to investigating toxic mechanisms in culture has been to examine directly the neoplastic response of keratinocytes to tumor initiators, promoters, and oncogenes (*15*), in some cases treating the animal skin before cultivation (*16*). This work often exploits the sensitivity of normal but not transformed murine cells to high calcium medium, where they terminally differentiate (*17*). Both normal and malignant human keratinocytes express xenobiotic metabolism (e.g., aryl hydrocarbon hydroxylase) in culture and consequently are sensitive to DNA damage and the mutagenic action of polycyclic aromatic hydrocarbons and related agents. Applicability of these cells to investigating mechanisms of mutagenesis and to screening of unknowns for mutagenic potency has been described (*18*).

A second approach to studying toxic mechanisms has been to examine the physiological modulation of keratinocyte differentiation and its disturbance by model agents. Acquisition of malignancy is generally a multistep process that can be set in motion by genetic damage, although alteration of developmental or other processes not clearly involving DNA damage can be important (*19*). Rare genetic events often appear to be rate limiting, but recent evidence points to a role for common cellular events in carcinogen-treated populations in enhancing the incidence or detection of malignant change (*20,21*). Moreover, the desirability of testing for chronic toxicity other than carcinogenesis is evident. Interference of model agents in the regulation of cellular processes or responses to signals from their microenvironment may be diagnostic for a variety of syndromes, possibly even at target sites other than stratified squamous epithelia.

For example, in view of their responsiveness to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (*22*), as well as to a number of endogenous effectors of differentiation, keratinocytes (including cell lines derived from neoplasias) provide a useful model in which to study the action of this agent. Pursuant to previous indications that the toxic action of TCDD may result from its interference with normal hormone action (*23*), a number of studies have now reported examples of this phenomenon (*24*). Malignant human keratinocytes appear defective in their ability to differentiate terminally (*25*). However, they can exhibit at confluence a moderately high level of keratinocyte transglutaminase, which is expressed in normal epidermis in the upper spinous layer (*7*), and a high keratin content. Hydrocortisone in the culture medium stimulates these markers 4- to 6-fold in the SCC-13 cell line. TCDD has little effect on their expression in the absence of added glucocorticoid, but it largely antagonizes the action of hydrocortisone when the cells are grown in the presence of both agents (*26*).

In contrast to SCC-13, the SCC-4 cell line shows relatively little keratinocyte character but does exhibit great sensitivity to vitamin A in induction of tissue transglutaminase to high levels (*11*). TCDD in the medium prevents the induction by retinoic acid or retinyl acetate. The concentration dependence of this action (EC_{50} = 20 pM) was virtually identical to that for induction of aryl hydrocarbon hydroxylase, compatible with mediation by the *Ah* receptor. The loss of cell sensitivity to vitamin A was also observed with 3-methylcholanthrene and benzo[a]pyrene but not pyrene or dibenzofuran. If, as generally assumed, the tumor promoting activity of TCDD is receptor mediated, this result suggests the promoting activity of carcinogenic polycyclic aromatic hydrocarbons may be at least in part conferred in the same way, independent of the generation of DNA-damaging electrophiles (*24*).

Materials and Methods

Cell Culture

SCC-12F2 keratinocytes were grown with 3T3 feeder layer support (*27*) in a 3:1 mixture of Dulbecco-Vogt Eagle's and Ham's F-12 media supplemented with fetal bovine serum (5%), hydrocortisone (0.4 μg/mL), transferrin (5 μg/mL), insulin (5 μg/mL), adenine (0.18 mM), triiodothyronine (20 pM), and antibiotics. The medium for the rat epithelial cells (*28,29*) also contained epidermal growth factor and cholera toxin (each approximately 10 ng/mL). The medium was changed at 3 to 4 day intervals. In experiments employing TPA or 3-MC (both from Sigma Chemical Co., St. Louis, MO), the agent was dissolved in dimethylsulfoxide and diluted from a stock solution into medium to give a final solvent concentration of 0.1%. Cultures were treated starting 1 to 2 days after inoculation and at each medium change with the indicated agent or solvent (control) and after approximately 2 weeks were fixed and stained with rhodanile blue (*30*). Rat epithelial cells were used for toxicity studies in passages 3 to 4 (approximately 30 to 40 generations).

Assays

SCC-12F2 cells were rinsed with isotonic saline, scraped into tubes with buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 1% Emulgen 911) and stored frozen. Thawed samples were disrupted by sonication and centrifuged at 100,000*g* for 45 min. To measure involucrin content, aliquots of the supernatant of appropriate dilution were incubated overnight with 0.06 mL of rabbit anti-involucrin antiserum (*2*), diluted 1:1500, transferred to a 96-well plate containing adsorbed, partially purified involucrin, incubated 30 min, and washed. Antibodies remaining bound to the wells were quantitated by treatment with protein A-alkaline phosphatase conjugate and subsequent incubation with *p*-nitrophenylphosphate. Absorbance values at 405 nm were measured using a Titertek Multiskan, and involucrin values were calculated from standard curves generated in parallel with samples of chromatographically purified involucrin (*8*). From the original crude sonicates, aliquots were assayed for transglutaminase in final volumes of 0.26 mL containing 0.5 mg of dimethylcasein, 100 mM Tris-HCl (pH 8.2), 4 mM $CaCl_2$, 0.4 mM EDTA, 5 mM dithioerythritol, 15 mM putrescine, and 0.5 μCi of ^{3}H-putrescine. After 30 min incubation at 37°C, protein-bound radioactivity was reco-

vered by trichloroacetic acid precipitation on glass fiber filters, rinsed with trichloroacetic acid and ethanol, and measured by scintillation counting (*11*). Measures of involucrin and transglutaminase were normalized to protein content of the sonicate assayed with bicinchoninic acid (*31*).

For assay of estrogen receptors, rat endometrial cells (passages 10–15) were treated with forskolin (dissolved in ethanol; final solvent concentration 0.05%) just as they reached confluence. After appropriate incubation, the cultures were rinsed in isotonic saline, scraped into tubes, and sonicated in buffer (10 mM Tris-HCl, pH 7.4, 20 mM $NaMoO_4$, 2 mM dithioerythritol). Samples were then adjusted to 0.4 M in KCl, stirred for 20 min at O°C, and centrifuged for 45 min at *100,000g*. Aliquots of the supernatant were adjusted to 1 nM in ^{3}H-estradiol (~ 100 Ci/mmole; New England Nuclear, Boston, MA) ± 200 nM unlabeled estradiol, incubated for 1 hr at 30°C, and the bound ligand recovered by hydroxyapatite precipitation and subsequent ethanol elution (*32*). Several other estradiol concentrations were employed in occasional experiments and gave essentially the same results. Specific binding was normalized to protein content in the soluble extract (typically 1 mg/mL) as measured by Coomassie G-250 dye binding (*33*).

Results

12-*O*-Tetradecanoylphorbol-13-Acetate

Cell lines derived from epidermal squamous carcinomas are generally insensitive to the toxic effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) compared to normal epidermal cells (*34,35*), as shown for SCC-13 in Figure 1. A special case is the human epidermal line SCC-12F2, immortal but not malignant (*27*), which is reduced in colony-forming efficiency by this agent (*34*). As illustrated in Figure 1, TPA produces a noticeable reduction in growth at 0.16 nM and largely prevents colony expansion at 1.6 to 16 nM. At the highest concentration tested, 160 nM, the growth rate was higher than at 1.6 to 16 nM. Close inspection through the culture microscope indicated that slow cell growth was continuing at the latter concentrations, but discrete colonies were not easily distinguishable by staining, probably due at least in part to the altered morphology of the cells and loss of their usual close association with each other. At higher inoculation densities, the treated cultures reached confluence and could be maintained for several weeks.

As found with other neoplastic keratinocyte lines such as SCC-13 (*36*), SCC-12F2 cells showed little differentiated character during log-phase growth with 3T3 feeder layer support. However, keratinocyte properties became evident as the cultures reached confluence, as shown in Figure 2. Transglutaminase activity rose from very low to appreciable values as the cells approached confluence, reaching approximately 10-fold higher levels than during mid-log-phase growth. Involucrin content increased 50-fold or more from basal levels, but this rise consistently occurred several days later than that of the transglutaminase. The ability of the cells to make cross-linked envelope structures upon stimulation by the ionophore X537A increased largely in concert with measured activity of the cross-linking enzyme. At early times, however, it was evident that the enzyme alone is insufficient to form the structures (evidently, substrates are lacking). Incorporation of involucrin likely stabilizes the structures, but is not required for their initial formation under these artificial conditions. Competence levels remained high as the cells were maintained at confluence, indicating that

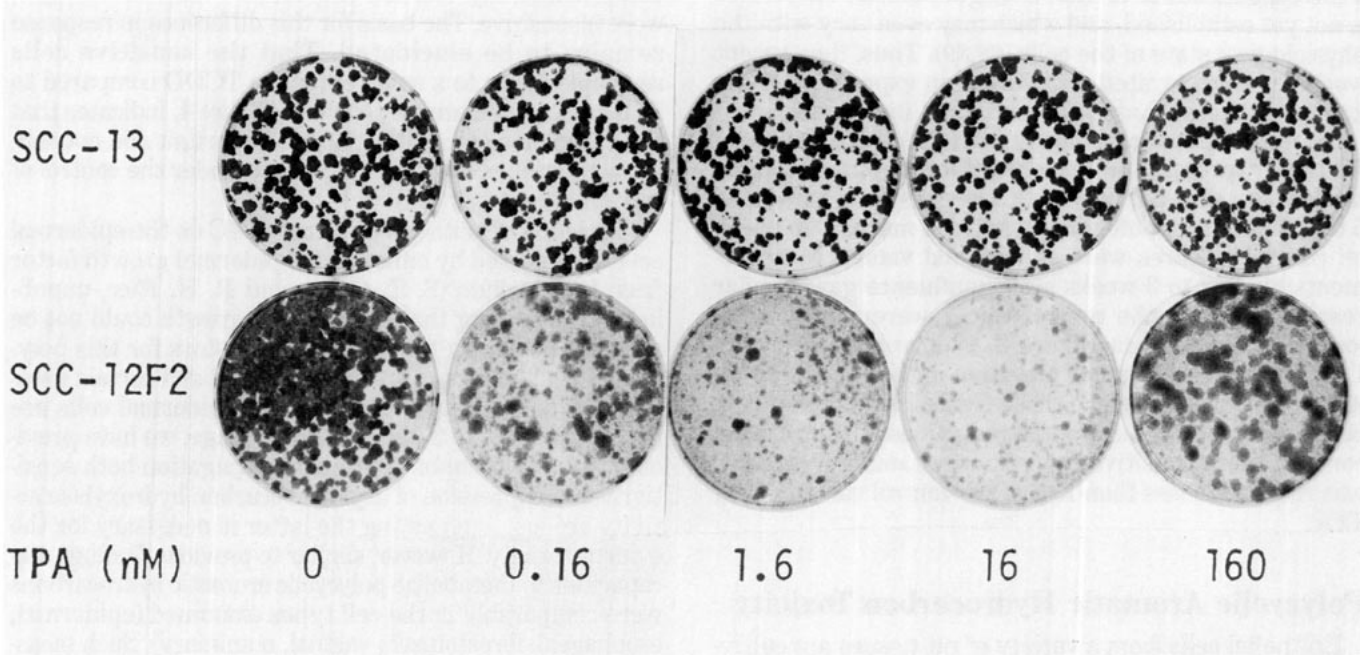

FIGURE 1. Effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) on growth of SCC-12F2 and SCC-13 cells.

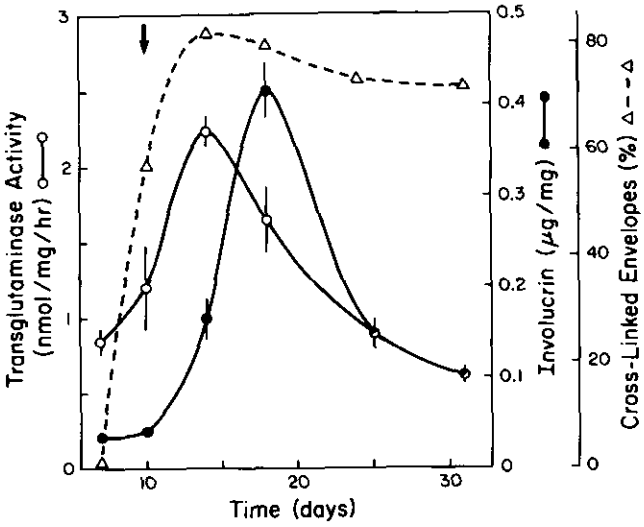

FIGURE 2. Kinetics of expression of transglutaminase, involucrin, and envelope competence in SCC-12F2 cells. Cultures inoculated with 10^5 cells/dish were harvested at the times indicated and assayed. Values shown are the mean and range of determinations in duplicate cultures. The range of envelope competence measurements, performed as previously described (*36*), was ±6%. The cultures became confluent on day 10 (↓). In other experiments, transglutaminase activity measured several days earlier than the first time point shown was approximately 10% of the maximum value attained shortly after confluence.

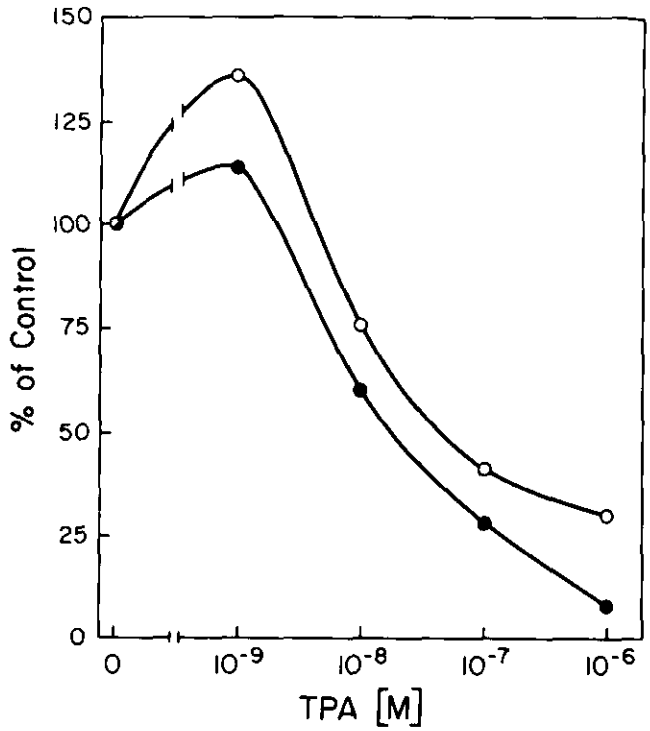

FIGURE 3. Effect of TPA on transglutaminase and involucrin expression in SCC-12F2 cells. Cultures grown as in Fig. 2 were treated with the indicated concentration of TPA and harvested 3 days after confluence for assay of transglutaminase (○) and involucrin (●).

necessary components were present in sufficient amounts despite the slow decline detected in both transglutaminase and involucrin. Similar observations have been made using SCC-9 cells, in which involucrin accumulation follows the rise in transglutaminase by at least a week (*37*). Measures of ionophore-stimulated envelope-forming ability evidently reflect a property of the cells for which a correspondence to certain transglutaminase substrates is not yet established, and which may even vary with the physiological state of the cells (*38,39*). Thus, the present work has concentrated specifically on expression of the known markers transglutaminase and involucrin.

Cultures treated chronically with TPA were examined for their expression of these keratinocyte differentiation markers, as shown in Figure 3. The cells were harvested 5 to 6 days after confluence, when the markers in parallel control cultures were at maximal values. Measurements made 2 to 3 weeks after confluence gave similar results, although the control values were considerably lower, as indicated in Figure 2. Cultures treated with 1 nM TPA showed a small elevation in the specific activity of both markers, while those treated with higher concentrations showed substantial reductions. The involucrin content, more sensitive than transglutaminase activity, was reduced to less than 10% of the control value at 1 μM TPA.

Polycyclic Aromatic Hydrocarbon Toxicity

Epithelial cells from a variety of rat tissues are cultivable with 3T3 feeder layer support (*28,29*). A survey of their sensitivities to inhibition of growth by 3-methylcholanthrene (3-MC) has shown striking differences, as summarized in Table 1. This agent produced its most powerful effect in rat epidermal and mammary epithelial cells, with a concentration dependence similar to that observed previously for epidermal cells (*40*), whereas bladder, prostate, thyroid, and endometrial cells, for example, were insensitive. The basis for this difference in response remains to be elucidated. That the sensitive cells responded only to a small extent to TCDD compared to 3-MC in the medium, as shown in Figure 4, indicates that the production of metabolites was important and not simply activation of whatever genes are under the control of the *Ah* receptor.

The action of neither TCDD nor 3-MC on the epidermal cells was altered by omission of epidermal growth factor from the medium (S. E. Crane and R. H. Rice, unpublished), indicating that inhibition of growth could not be ascribed primarily to the loss of receptors for this polypeptide that occurs in human epidermal and certain keratinocyte lines (*41,42*). Although rat epidermal cells are highly sensitive to 3-MC in early passage, we have previously found that upon continued propagation both sensitivity and expression of aryl hydrocarbon hydroxylase activity are lost, suggesting the latter is necessary for the evident toxicity. However, similar to previous findings, the capacities to metabolize polycyclic aromatic hydrocarbons were comparable in the cell types examined (epidermal, esophageal, forestomach, vaginal, mammary). Such measurements in growing or confluent cultures have not distinguished sensitive and insensitive cell types, implicat-

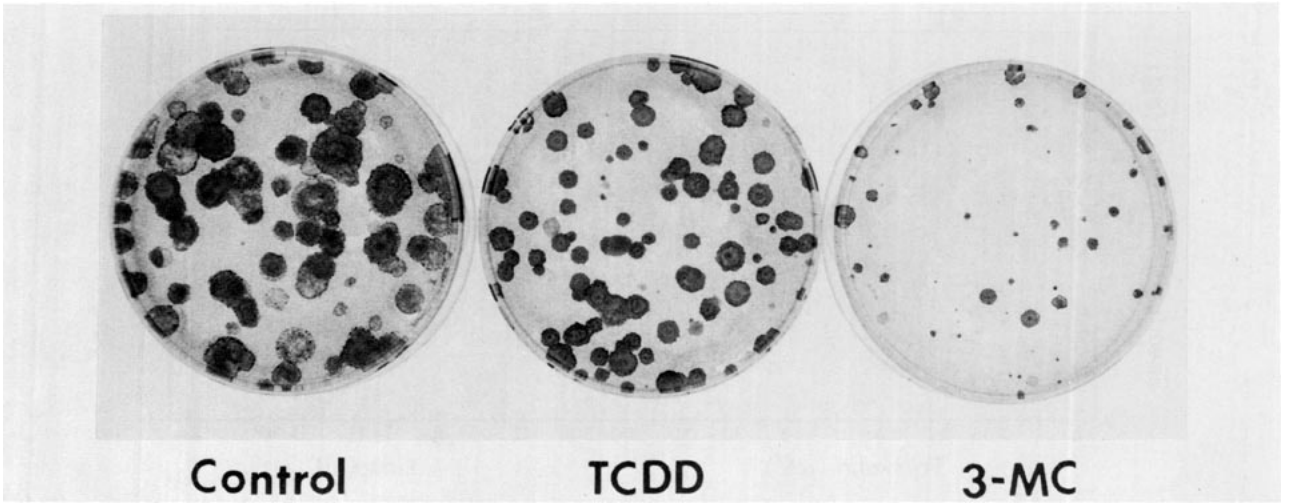

FIGURE 4. Sensitivity of rat mammary epithelial cells to growth inhibition by TCDD (10 nM) and 3-MC (2 μM).

ing other factors as well in determining the response (*40*, S. E. Crane and R. H. Rice, unpublished data). The actual metabolites have not been analyzed, leaving open the possibility of critical differences in hydroxylation, conjugation, DNA repair, quinone or radical scavenging, or even cooxidation with arachidonic acid upon membrane damage. In regard to the latter possibility, growth of the rat mammary cells in the presence of cyclooxygenase or lipoxygenase inhibitors indomethacin or BW755C (up to 3 μM or 1 μM, respectively, above which the agents were toxic alone) did not reduce the toxicity of 0.2 to 2 μM benzo[a]pyrene in the growth medium. Moreover, addition of 1 μM arachidonic acid to the medium did not inhibit the cell growth alone or in the presence of benzo[a]pyrene (E. Choi and R. H. Rice, unpublished).

Estrogen Receptor Modulation

Rat endometrial cells grown with feeder layer support express estrogen receptors at a modest level (*32*), a stable characteristic even after approximately 200 generations. Additives to the culture medium such as hydrocortisone and epidermal growth factor had little or no observable effect on receptor content. However, agents increasing production of cAMP (cholera toxin, forskolin) were found to raise substantially the receptor level without significant effect on affinity for the ligand. As shown in Figure 5, forskolin at high concentration (100 μM) increased the receptor level by 3- to 6-fold with an EC_{50} of approximately 3 μM (left panel). Forskolin has also been reported to increase glucocorticoid receptor levels 2-fold in murine lymphoma cells without altering affinity for the ligand (*43*). Steroid receptors are known to be subject to phosphorylation, which modulates their ability to bind ligand and may alter their affinity for DNA acceptor sites (*44*). The estrogen receptor is a substrate for a calmodulin-dependent kinase that phosphorylates a critical tyrosine residue (*45*). The present findings indicate forskolin addition does not increase receptor number simply by stimulating cAMP-dependent receptor phosphorylation. Its action takes approximately 6 hr for maximal effect (Figure 5, right panel) and is prevented by cycloheximide, indicative of a requirement for protein synthesis (*32*).

Physiological agents raising intracellular cAMP, including follicle-stimulating hormone and prostaglandins E_1 or E_2, are known to be effective in modulating functions of estrogen responsive cells of the female genital tract (*46*). In a search for endogenous effectors that might be of physiological relevance, cultures were treated with crude preparations of several peptide hormones obtained from the National Hormone and Pituitary Program. Standard preparations of ovine (follicle stimulating hormone), porcine (follicle stimulating hormone, leutinizing hormone), and rat (growth hormone, prolactin, hypothalamic extract) origin employed under various conditions did not materially alter the receptor level. In some experiments, treatment of the cultures with prostaglandins E_1 or E_2 led to a 2- to 3-fold increase in receptor level over a 4 to 6 hr time period (prostaglandins B_1, I_2 and $F_{2\alpha}$ were virtually inactive), but this finding was not readily reproducible, perhaps due to variable endogenous prostaglandin production by the epithelial or the few remaining 3T3 cells.

Table 1. Inhibition of colony expansion.

Epithelial origin	Inhibitory activity[a] TCDD	3-MC
Epidermal	+	+++
Mammary	+	+++
Seminal vesicle	+	++
Forestomach	+	++
Pituitary	+	+
Esophageal	–	–
Vaginal	–	–
Prostate	–	–
Thryoid	–	–
Bladder	[b]	–
Endometrial	[b]	–

[a]The degree of colony growth inhibition in the presence of TCDD (10 mM) or 3-MC (4 μM) was judged in comparison with parallel solvent-treated controls as none (–) or mild (+) to severe (+++).
[b]Not tested.

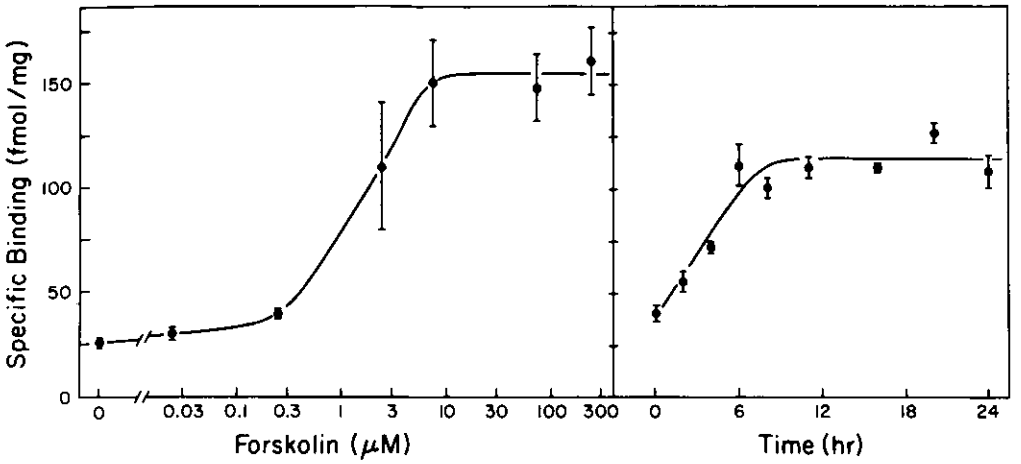

FIGURE 5. Estrogen receptor stimulation by forskolin. Rat endometrial cells were treated with forskolin at the indicated concentrations for 18 hr (left panel) or at 100 μM for the indicated times (right panel). The cultures were then harvested and assayed for receptor content.

Discussion

Treatment of cultured keratinocytes from normal mouse or human epidermis with TPA induces a state resembling terminal differentiation in a majority of the cells, although a substantial fraction of transiently resistant cells is evident corresponding to the germinative population (*34,35,47*). This finding has suggested that sensitivity to TPA requires some differentiated function lacking in basal cells and certain human squamous carcinoma lines. However, such carcinoma cells can express considerable differentiated function upon reaching confluence (*36*), suggesting they might show a sensitivity to TPA under optimal circumstances. Growth of the malignant cell line SCC-9 with 1 nM to 1 μM TPA in the medium gave no hint of toxicity or stimulation of differentiation. Nevertheless, expression of involucrin and particulate transglutaminase in confluent cultures was greatly suppressed (*37*). In the present experiments, it is clear that the latter phenomenon occurs in SCC-12F2, a premalignant line in which TPA does inhibit cell growth. Whether a transient stimulation of expression of the two markers occurs after initial TPA treatment has not yet been established.

Upon chronic treatment of SCC-12F2 (and SCC-9) cultures, the TPA concentration dependence shows that involucrin is considerably more sensitive to suppression than transglutaminase. Whether this phenomenon represents an effect on transcription remains to be seen. The keratinocyte transglutaminase is subject to phosphorylation, which is stimulable 5-fold by treatment of cultured normal epidermal cells for several hours with TPA (R. Chakravarty and R. H. Rice, unpublished). Thus, post-translational modification(s) could account for some alteration in measured activity. Moreover, involucrin is quite sensitive to proteolytic degradation and could be subject to rapid depletion as a consequence of protease activation. Indeed, protein kinase C is dramatically depleted in a variety of cell types, including A431 malignant human keratinocytes (*48*), as well as in mouse skin (*49*), perhaps due to enhanced proteolysis (*50*). The possibility that TPA-mediated production of reactive oxygen species (*51,52*) may contribute to this differentiation suppression has not been ruled out, however. If protein kinase C ordinarily mediates keratinocyte differentiation, suggested by its observed stimulation of differentiated properties in cultured normal keratinocytes, then its depletion could be responsible for the suppressive effect in these chronically treated cells. Regardless of this hypothesis, a biphasic growth inhibition response induced by TPA as in SCC-12F2 has been reported in a number of cell types (*53*). In some cases (such as this), perhaps a drastic depletion of protein kinase C at the highest concentrations of TPA employed could release the cells from a growth inhibitory influence of the kinase activity present at lower concentrations.

Although keratinocytes from various anatomic sites have much in common, they retain and under appropriate circumstances display intrinsic differences in their differentiation program (*54*), perhaps arising in part as a consequence of their developmental pathways. In addition to pharmacokinetic factors, these differences are likely to result in distinctive responses to physiological and pathological conditions. The striking contrast in the response of cultured rat epithelial cells to the polycyclic aromatic hydrocarbon 3-MC suggests these intrinsic differences could have major influences on carcinogen target specificity, for example. It is not clear at present how relative toxicity (growth inhibition) may relate to carcinogenicity in this instance. One possibility, similar to the selection scheme proposed for preneoplastic lesions of the rat liver (*55*), is that epidermal cells metabolizing the agent are prevented from growing. Those variants arising which lack such metabolic capability may then be selectively provided with the opportunity to replace the normal cells. Variants lacking aryl hydrocarbon hydroxylase that are not sensitive to 3-MC toxicity do arise in epidermal cell populations even without 3-MC treatment upon continued passage (*28*). Alternately, one could imagine that continued carcinogen metabolism by the esophageal cells without toxicity might permit a greater burden of DNA

damage to accumulate, thereby enhancing the rate of progression to malignancy. Further information on the degree of DNA damage suffered by these cells and the effect of treatment on their growth properties may help choose among such possible scenarios. Insensitivity to 3-MC and B[a]P but not to dimethylbenzanthracene, a more potent carcinogen at this target site *in vivo*, has been noted previously in primary cultures of rat tracheal epithelial cells (*56*). These cells express in surface culture the metabolic capabilities observed in organ culture (*57*). While rodent epidermis and mammary epithelium are well-known target sites for polycyclic aromatic hydrocarbon carcinogenesis, few studies have examined systematically the relative target sensitivity of these and other sites. If the observed sensitivity in culture reflects that in the animal, examination of its biochemical basis may be of utility in improving extrapolation among epithelia and perhaps species.

To what degree differentiated properties of the cultured rat cells correspond to the epithelia of origin remains to be explored in detail. Those derived from stratified squamous epithelia presumably retain the most normal function, although probably resembling the hyperproliferative state observed in human epidermal cells (*58*). With respect to other cell types, the adoption of features such as high keratin content and envelope-forming ability (*29*) and expression of a particulate transglutaminase (*14*) indicate a reprogramming resembling, but not necessarily identical to (*59*), squamous metaplasia. In any case, the culture phenotype appears to approximate behavior within the cellular repertoire *in vivo* under conditions of stress (hormonal imbalance, vitamin deprivation, physical trauma, neoplastic influence). Squamous metaplasia is inducible even in the rodent mammary epithelium (*60*) and is seen in human mammary tissue (*61*). New biochemical properties may well influence the target susceptibility of these cells to exogenous agents or alter the ensuing pathology. Loss of characteristic specialized function may be only partial, with enough remaining to permit study of important normal processes. Thus, the responsiveness of estrogen receptor content in the rat endometrial cells to agents elevating intracellular cAMP suggests possible usefulness in studying the mechanism of this phenomenon and consequences of its disturbance. Enhanced estrogen responsiveness of the cells by cAMP elevation may merit exploration, especially in conjunction with alterations in the growth substratum that have permitted maintenance of normal function in cultured mammary and tracheal epithelial cells (*62,63*). In addition, since the adenyl cyclase/phosphodiesterase pathway is sensitive to perturbation by exogenous agents, the cells may provide a suitable model for examining certain types of reproductive toxicity.

As these results demonstrate, epithelial cell culture models can be instrumental in elucidating physiological and biochemical bases for differences in regulation and response among tissue types and species. Further investigations in these directions, in conjunction with *in vivo* observations, could ultimately aid in prediction of human responses to new or uncharacterized toxic substances.

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